

Targeted disruption of the $\alpha 1,3$ -galactosyltransferase gene in cloned pigs

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Galactose- $\alpha 1,3$ -galactose ($\alpha 1,3$ Gal) is the major xenoantigen causing hyperacute rejection in pig-to-human xenotransplantation. Disruption of the gene encoding pig $\alpha 1,3$ -galactosyltransferase ($\alpha 1,3$ GT) by homologous recombination is a means to completely remove the $\alpha 1,3$ Gal epitopes from xenografts. Here we report the disruption of one allele of the pig $\alpha 1,3$ GT gene in both male and female porcine primary fetal fibroblasts. Targeting was confirmed in 17 colonies by Southern blot analysis, and 7 of them were used for nuclear transfer. Using cells from one colony, we produced six cloned female piglets, of which five were of normal weight and apparently healthy. Southern blot analysis confirmed that these five piglets contain one disrupted pig $\alpha 1,3$ GT allele.

Galactose- $\alpha 1,3$ -galactose ($\alpha 1,3$ Gal) epitopes are a common carbohydrate structure on the cell surface of almost all mammals with the exception of humans, apes, and Old World monkeys¹. Synthesis of the $\alpha 1,3$ Gal epitope is catalyzed by the enzyme $\alpha (1,3)$ galactosyltransferase ($\alpha 1,3$ GT)². Humans do not have a functional copy of the $\alpha 1,3$ GT gene, and hence do not show $\alpha 1,3$ Gal surface expression. The presence of the $\alpha 1,3$ Gal antigen on the surface of pig cells and tissues is the major cause of hyperacute rejection (HAR) in pig-to-human xenotransplantation²⁻⁴. It has been reported that, in humans, up to 1% of the total circulating IgG is anti- $\alpha 1,3$ Gal natural antibody⁵. A number of strategies have been used to reduce or eliminate $\alpha 1,3$ Gal-induced HAR. These methods²⁻⁴ include overexpression of $\alpha 2,3$ -sialyltransferase or $\alpha 1,2$ -fucosyltransferase in pig cells to compete with $\alpha 1,3$ GT; treatment of pig organs with α -galactosidase to remove surface $\alpha 1,3$ Gal epitopes; expression of complement inhibitor genes, such as human decay-accelerating factor (DAF), in transgenic pig organs to suppress the complement reaction; and temporary depletion of natural anti- $\alpha 1,3$ Gal antibody from recipients before transplantation. All these methods only partially or temporarily remove the $\alpha 1,3$ Gal from the surface of the xenografts, however, and the residual $\alpha 1,3$ Gal molecules are still sufficient to activate the complement cascade and cause destruction of the grafts²⁻⁴. Complete elimination of $\alpha 1,3$ Gal epitopes from the donor organs should be achievable by removal of the $\alpha 1,3$ GT gene. $\alpha 1,3$ GT knockout mice have been made by a number of groups⁶⁻⁷. When tissues from these mice are exposed to human serum, they bind substantially less human anti-Gal xenoantibody than do tissues from normal mice, resulting in a significant decrease in human complement activation⁶.

The cloning of sheep⁸, goat⁹, cattle¹⁰, and pigs¹¹ by somatic cell nuclear transfer provides an alternative means of disrupting or deleting genes in mammals other than mice. The production of cloned sheep with targeted insertions at the ovine $\alpha 1(I)$ -procollagen (*COL1A1*) locus showed that viable animals can be produced via nuclear transfer with gene-targeted cultured fibroblasts¹². The

$\alpha 1,3$ GT gene has recently been successfully deleted in sheep fibroblasts and in fetuses cloned from targeted cells¹³. Although no viable animals resulted, these experiments showed that it is feasible to disrupt the $\alpha 1,3$ GT gene using nuclear transfer techniques in livestock. Lai *et al.* have recently described the disruption of one allele of the $\alpha 1,3$ GT gene in pig fibroblasts and in four live piglets cloned from these cells¹⁴. However, the only evidence of gene targeting offered in this report was PCR analysis of recombination junctions. Here we present genomic Southern blot analyses showing successful disruption of one copy of the $\alpha 1,3$ GT gene in cultured male and female porcine fetal fibroblasts. To date we have produced five apparently healthy $\alpha 1,3$ GT knockout female piglets by nuclear transfer.

Results and discussion

Because the $\alpha 1,3$ GT gene is expressed well in porcine fetal fibroblasts (PPL Therapeutics, unpublished data), it is possible to enrich for homologous recombination events using a promoter-trap knockout-vector strategy¹². Two similar knockout vectors, pPL654 and pPL657, were constructed from isogenic DNA of SLA1-10 and PCFF4-2 cells, respectively, by inserting an *IRES-neo*-poly A cassette into the 5' end of exon 9 (Fig. 1A). Because the majority of the coding region of the pig $\alpha 1,3$ GT gene, including the sequences encoding the catalytic domain, is located in exon 9, successful targeting using these vectors is expected to result in functional inactivation of the gene⁶⁻⁷.

Four different early-passage (P2 or P3) primary porcine fetal fibroblasts cell lines were used for transfection: the male cell lines SLA1-10, PCFF4-2, and PCFF4-3 and the female cell line PCFF4-6. SLA1-10 cells were transfected with the isogenic vector pPL654, and PCFF4-2 cells were transfected with the isogenic vector pPL657. The PCFF4-3 and PCFF4-6 cell lines were derived from sibling fetuses of the fetus used to derive PCFF4-2, and therefore were transfected with the pPL657 vector. G418-resistant colonies were screened by 3' PCR with neo442S (a sequence from the 3' end of *neo*) and α GTE9A2 (a sequence from the 3' end of exon 9 in sequences located outside the 3' recombination

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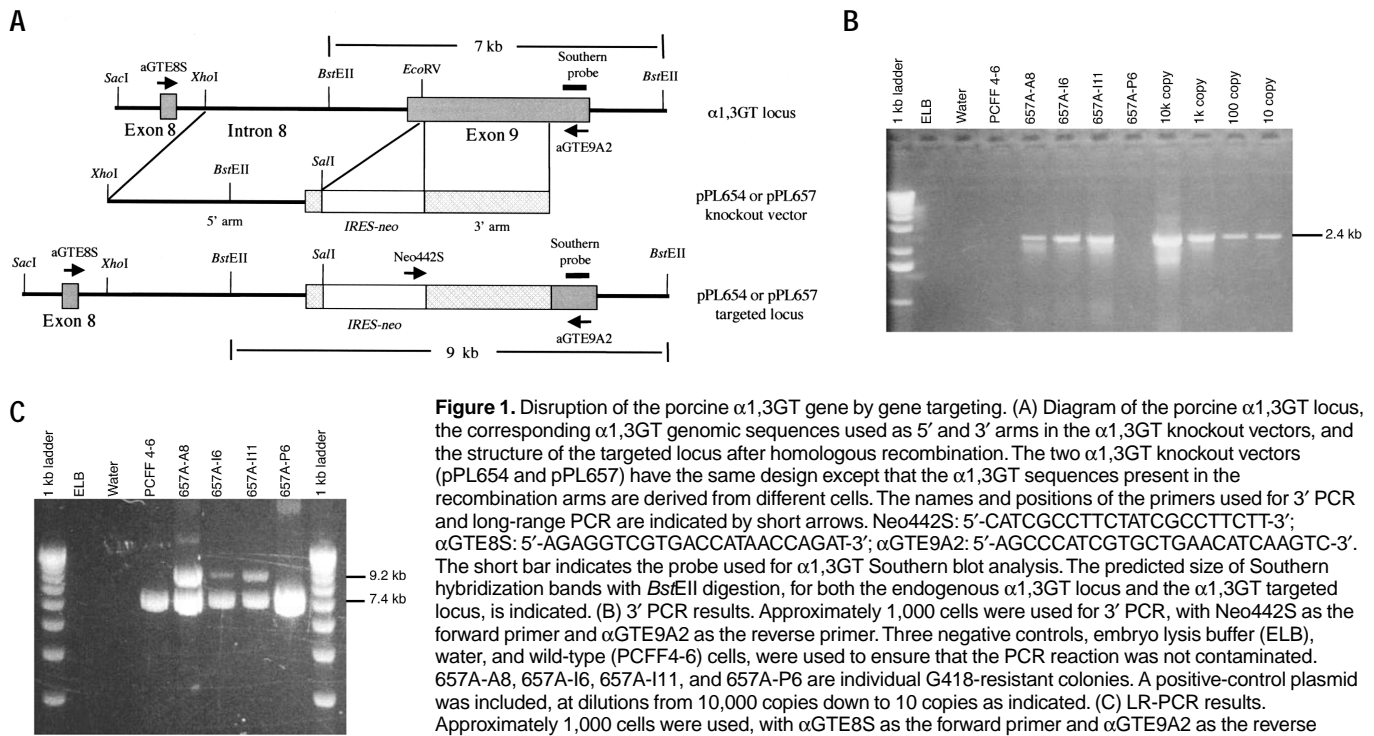


Figure 1. Disruption of the porcine $\alpha 1,3GT$ gene by gene targeting. (A) Diagram of the porcine $\alpha 1,3GT$ locus, the corresponding $\alpha 1,3GT$ genomic sequences used as 5' and 3' arms in the $\alpha 1,3GT$ knockout vectors, and the structure of the targeted locus after homologous recombination. The two $\alpha 1,3GT$ knockout vectors (pPL654 and pPL657) have the same design except that the $\alpha 1,3GT$ sequences present in the recombination arms are derived from different cells. The names and positions of the primers used for 3' PCR and long-range PCR are indicated by short arrows. Neo442S: 5'-CATCGCCTTCTATCGCCTTCTT-3'; $\alpha GTE8S$: 5'-AGAGTCTCGTGACCATAACCAGAT-3'; $\alpha GTE9A2$: 5'-AGCCCATCGTGTGCAACATCAAGTC-3'. The short bar indicates the probe used for $\alpha 1,3GT$ Southern blot analysis. The predicted size of Southern hybridization bands with *BstEII* digestion, for both the endogenous $\alpha 1,3GT$ locus and the $\alpha 1,3GT$ targeted locus, is indicated. (B) 3' PCR results. Approximately 1,000 cells were used for 3' PCR, with Neo442S as the forward primer and $\alpha GTE9A2$ as the reverse primer. Three negative controls, embryo lysis buffer (ELB), water, and wild-type (PCFF4-6) cells, were used to ensure that the PCR reaction was not contaminated. 657A-A8, 657A-I6, 657A-I11, and 657A-P6 are individual G418-resistant colonies. A positive-control plasmid was included, at dilutions from 10,000 copies down to 10 copies as indicated. (C) LR-PCR results. Approximately 1,000 cells were used, with $\alpha GTE8S$ as the forward primer and $\alpha GTE9A2$ as the reverse primer. ELB, water, and pCFF4-6 cells were used as negative controls. 657A-A8, 657A-I6, 657A-I11, and 657A-P6 are individual G418-resistant colonies.

arm) as forward and reverse primers (Fig. 1A). Thus, only through successful targeting at the $\alpha 1,3GT$ locus would the expected 2.4-kb PCR product be obtained. From a total of seven transfections in four different cell lines, 1,105 G418-resistant colonies were picked, of which 100 (9%) were positive for $\alpha 1,3GT$ gene disruption in the initial 3' PCR screen (range 2.5–12%; Table 1). Figure 1B shows the 3' PCR results for a representative group of G418-resistant colonies. Colonies 657A-A8, 657A-I6, and 657A-I11 showed the expected 2.4-kb band, whereas control PCFF4-6 cells and another G418-resistant colony, 657A-P6, did not. A portion of each 3' PCR-positive colony was frozen immediately in several small aliquots for future use in nuclear transfer experiments, and the rest of the cells were expanded for long-range PCR (LR-PCR) and Southern blot analysis.

From our and others' experience^{15–16}, we expected that DNA analysis by PCR, or mRNA analysis by reverse transcription PCR, to detect recombination junctions would be prone to generating false-positive results. Therefore, to further confirm successful targeting at the $\alpha 1,3GT$ locus, we carried out an LR-PCR experiment encompassing the entire targeted region. The LR-PCR covered the 7.4-kb $\alpha 1,3GT$ genomic sequence from exon 8 to the end of exon 9, with both primers ($\alpha GTE8S$ and $\alpha GTE9A2$) located outside the recombination region

(Fig. 1A). The control PCFF4-6 cells and the 3' PCR-negative colony, 657A-P6, showed only the endogenous 7.4-kb band from the wild-type $\alpha 1,3GT$ locus (Fig. 1C). In contrast, three of the 3' PCR-positive colonies, 657A-A8, 657A-I6, and 657A-I11, showed both the 7.4-kb endogenous band and a new band of 9.2 kb, the size expected for targeted insertion of the 1.8-kb *IRES-neo* cassette into the $\alpha 1,3GT$ locus. As some 3' PCR-positive signals may come from PCR artifacts, the LR-PCR assay is crucial to confirm successful knockout events. As evidence of this fact, only 30% of the 3' PCR-positive colonies could be confirmed by LR-PCR (Table 1).

Approximately half (17/30) of the LR-PCR-positive colonies were successfully expanded to yield enough cells (1×10^6) for Southern blot analysis. We expected that the colonies would be heterozygous for knockout at the $\alpha 1,3GT$ locus and thus would have one normal copy and one disrupted copy of the $\alpha 1,3GT$ gene. With *BstEII* digestion, the $\alpha 1,3GT$ knockout cells should show two bands: a 7-kb band of the size expected for the endogenous $\alpha 1,3GT$ allele and a 9-kb band characteristic of insertion of the *IRES-neo* sequences at the $\alpha 1,3GT$ locus (Figs. 1A, 2). Southern blot analysis confirmed knockout of the gene in all 17 LR-PCR-positive colonies. The same membranes were re-probed with sequences specific for *neo*, and the 9-kb band was detected with the *neo* probe (data not shown), confirming the targeted insertion of the *IRES-neo* cassette at the disrupted $\alpha 1,3GT$ locus. Table 1 summarizes the results of transfection, 3' PCR, LR-PCR, and Southern blot analysis. Recombination frequencies were highest in the PCFF4-2 and PCFF4-6 cell lines. On the basis of the LR-PCR results, the overall $\alpha 1,3GT$ knockout rate in G418-resistant colonies was 6% for PCFF4-2 cells, 3% for PCFF4-6 cells, and 0.5% for PCFF4-3 cells. The fact that PCFF4-2 cells gave the highest recombination frequency may be related to isogenicity

Table 1. Summary of 3' PCR, LR-PCR, and Southern analysis results of G418-resistant colonies

Cells (sex)	Knockout vectors	No. of G418 ^R colonies	No. of 3' PCR ⁺ colonies (%)	No. of LR-PCR ⁺ colonies (%)	No. of Southern ⁺ colonies (%)
SLA1-10 (M)	pPL654	127	4 (3%)	0	0
PCFF4-2 (M)	pPL657	179	22 (12%)	11 (6%)	2 (1%)
PCFF4-3 (M)	pPL657	200	5 (2.5%)	1 (0.5%)	1 (0.5%)
PCFF4-6 (F)	pPL657	599	69 (11.5%)	18 (3%)	14 (2%)

Results for SLA1-10, PCFF4-2, and PCFF4-6 cells are from two individual transfections for each cell; result for PCFF4-3 cells is from one transfection.

of these cells with the pPL657 GT knockout vector. When compared with PCFF4-2 cells, PCFF4-6 cells had a very similar knockout efficiency even though they were transfected with vector made from non-isogenic DNA. As both the PCFF4-2 and PCFF4-6 cell lines were derived from sibling fetuses of the same pregnancy, it is possible that they share a common allele.

We used seven Southern blot-confirmed $\alpha 1,3\text{GT}$ -knockout single colonies for nuclear transfer. All cells used for nuclear transfer were from the aliquots that had been frozen immediately after the initial 3' PCR screening. The karyotype of each colony was checked; all had chromosome numbers in a range similar to that of freshly isolated porcine fetal fibroblast cells (~70% of spreads with 38 chromosomes). On average, approximately 150 reconstructed nuclear-transfer embryos were transferred to the oviducts of each estrus-synchronized recipient female. All seven colonies used for nuclear transfer resulted in very high initial pregnancy rates at day 25 (50%–86%) (Table 2). However, all pregnancies established from colonies 657A-A8 and 657A-F12 were lost between days 25 and 45. In contrast, the other five colonies resulted in pregnancy rates in excess of 50% at day 45.

One spontaneously aborted day 38 fetus from a 657A-A8 nuclear transfer recipient was recovered. LR-PCR and Southern blot analysis confirmed that the fetus contained a disrupted $\alpha 1,3\text{GT}$ locus (data not shown). Southern blot results from 657A-I11 cells showed that the 9-kb knockout band was less intense than the 7-kb endogenous $\alpha 1,3\text{GT}$ band, indicating that this was most likely a mixed colony containing both wild-type and heterozygous knockout cells. There was some concern that fetuses derived from wild-type cells in the mixed colony could affect the development of, or outcompete, *in utero* fetuses derived from the $\alpha 1,3\text{GT}$ -knockout cells. To test this, we terminated by hysterectomy a day 32 pregnancy derived from nuclear transfer with 657A-I11 cells. Seven fetuses were recovered, of which six were of normal size and one substantially smaller. Southern blot analysis showed that six of the seven fetuses contained a disrupted $\alpha 1,3\text{GT}$ locus (Fig. 2A). Notably, it was the smaller fetus (fetus no. 2) that was wild type, and all six normal-sized fetuses contained an $\alpha 1,3\text{GT}$ knockout allele. These results suggested that there was no discrimination against the heterozygous $\alpha 1,3\text{GT}$ knockout fetuses *in utero*.

Six live piglets derived from the 657A-I11 cells were born on December 25, 2001. Five were of normal size and weight (Fig. 3); one (no. 2) was stunted, weighing less than 1 pound. Southern blot analysis indicated that five of the six offspring were $\alpha 1,3\text{GT}$ heterozygous knockouts (Fig. 2B). Again, the one negative (wild-type) piglet was the underdeveloped runt. These results, when considered along with the analysis of the seven day 32 fetuses from 657A-I11 cells, suggested that the colony 657A-I11 was indeed a mixed population contaminated with wild-type cells. All fetuses and offspring obtained from the $\alpha 1,3\text{GT}$ knockout cells in the 657A-I11 population were developmentally normal. Physical examination of the five knockout piglets at one month of age found no abnormalities. This contrasts with the

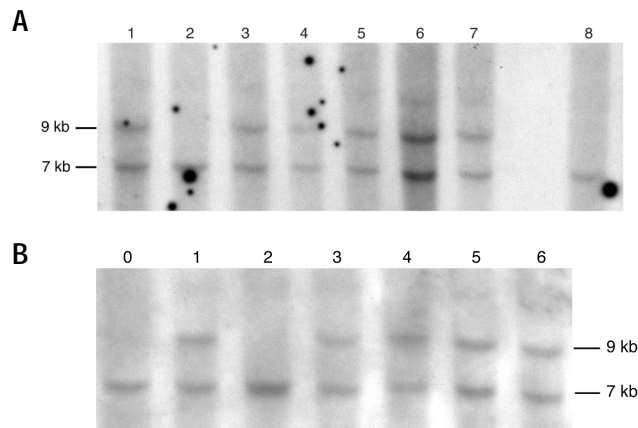


Figure 2. Southern blot analysis of $\alpha 1,3\text{GT}$ gene knockout fetuses and piglets. (A) Southern analysis of DNA from seven day 32 fetuses. Lane 1–7 are *Bst*EII-digested genomic DNA from seven day 32 fetuses derived from 657A-I11 cells. Lane 8 contains normal pig DNA digested with *Bst*EII as a negative control. The 7-kb band represents the endogenous $\alpha 1,3\text{GT}$ gene and the 9-kb band the disrupted $\alpha 1,3\text{GT}$ locus. (B) Southern analysis of DNA from six piglets. Lane 0 is the normal pig DNA digested with *Bst*EII. Lanes 1–6 are *Bst*EII-digested DNA from six piglets cloned from 657A-I11 cells. The 7-kb band represents the endogenous $\alpha 1,3\text{GT}$ gene and the 9-kb band represents the disrupted $\alpha 1,3\text{GT}$ locus.

report by Lai *et al.*¹⁴, in which only four of seven piglets survived for more than a month and three of the surviving piglets had mild physical abnormalities. These differing outcomes may have been due to many factors, including different pig breeds, different condition of cells used for nuclear transfer, and different embryo manipulation methods.

We have produced apparently healthy heterozygous $\alpha 1,3\text{GT}$ knockout piglets by nuclear transfer. We have an additional 16 ongoing second- and third- trimester pregnancies beyond day 45 from two female and three male $\alpha 1,3\text{GT}$ knockout colonies (Table 2). We expect that most will go to term as we have never lost any pregnancies of cloned pigs after 45 days of gestation (data not shown). The next step is to obtain homozygous pigs with both $\alpha 1,3\text{GT}$ alleles inactivated. This could be done either through natural breeding of male and female heterozygous knockout animals or through gene targeting with heterozygous knockout cells to disrupt the second $\alpha 1,3\text{GT}$ allele before a second round of nuclear transfer. As we already have six lines of early-passage heterozygous $\alpha 1,3\text{GT}$ gene-disrupted fetal fibroblasts, obtained from the day 32 657A-I11 fetuses, it will probably be considerably faster to create the second knockout in these cells *in vitro* and obtain homozygous knockout animals by nuclear transfer. Natural breeding to homozygosity will also be used, but this method will take considerably longer because of the gestation time of the male knockout clones *in utero* and the time to sexual maturity.

Live births have been reported in cattle from recloning experiments

that used fibroblasts obtained from cloned fetuses¹⁷. Recloning experiments by our group, using wild-type porcine fetal fibroblasts derived from a day 40 cloned fetus, have shown an 80% pregnancy rate at day 45 (PPL Therapeutics, unpublished data). These data suggest that it will be feasible to obtain homozygous $\alpha 1,3\text{GT}$ knockout pigs by a second knockout and recloning

Table 2. Summary of nuclear transfer results from $\alpha 1,3\text{GT}$ knockout primary fibroblast cells

Nuclear donor cell line	PCFF4-6	PCFF4-6	PCFF4-6	PCFF4-6	PCFF4-2	PCFF4-2	PCFF4-3
Cell clone	657A-I11	657A-A8	657A-F12	657A-I6	657F-J10	657F-C11	657B-K8
Sex	F	F	F	F	M	M	M
Embryos transferred to recipients	1097	825	591	976	1009	775	1105
Recipients	7	5	4	6	6	4	7
Pregnancies at day 25	6	3	2	5	5	2	6
Pregnancies at day 45	3 ^a	0	0	4	4	2	4 ^b
Piglets at birth ^c	6	0	0	Pending	Pending	Pending	Pending

^aBased on 6 recipients since one day 32 pregnancy was terminated for fetal fibroblast isolation. ^bBased on 6 recipients since one day 39 pregnancy was terminated for fetal fibroblast isolation. ^cAs dated on January 25, 2002. Six piglets were born from one 657A-I11 recipient on December 25, 2001. Another 16 recipients beyond day 45 of pregnancy are due after January 25, 2002.



Figure 3. Five $\alpha 1,3GT$ gene knockout piglets at 2 weeks of age.

strategy. In mice, the homozygous knockout of $\alpha 1,3GT$ gene is not an embryonic lethal mutation, although such mice have developed cataracts⁶. Pig cells express significantly more $\alpha 1,3Gal$ epitopes on their surface than do mouse cells, and it has therefore been proposed that $\alpha 1,3GT$ may have some additional, unknown role in pigs¹⁸. Although heterozygous $\alpha 1,3GT$ knockout pigs are developmentally normal, it is not known if complete deletion of both alleles of $\alpha 1,3GT$ will be more (or less) problematic in pigs than in mice.

Complete removal of the $\alpha 1,3Gal$ epitope, the major xenoantigen, combined with transgenic expression of complement regulatory proteins should prevent the hyperacute rejection of pig xenografts even in the presence of a low background of non- $\alpha 1,3Gal$ xenoantigens. The success of xenotransplantation will also depend on risk assessment of safety factors such as porcine endogenous retroviruses and on the development of strategies that address delayed vascular and T-cell-mediated rejection. Together, these approaches may provide a near-term solution to the chronic shortage of human organs (such as heart and kidneys) and valuable tissues such as insulin-producing islet cells.

Experimental protocol

Isolation and transfection of primary porcine fetal fibroblasts. PCFF4-1 to PCFF4-10 fetal fibroblast cells were isolated from 10 fetuses of the same pregnancy at day 33 of gestation. After removing the head and viscera, fetuses were washed with Hanks' balanced salt solution (HBSS; Gibco-BRL, Rockville, MD), placed in 20 ml of HBSS, and diced with small surgical scissors. The tissue was pelleted and resuspended in 50-ml tubes with 40 ml of DMEM and 100 U/ml collagenase (Gibco-BRL) per fetus. Tubes were incubated for 40 min in a shaking water bath at 37°C. The digested tissue was allowed to settle for 3–4 min and the cell-rich supernatant was transferred to a new 50-ml tube and pelleted. The cells were then resuspended in 40 ml of DMEM containing 10% fetal calf serum (FCS), 1 \times nonessential amino acids, 1 mM sodium pyruvate (Gibco-BRL), and 2 ng/ml basic fibroblast growth factor (bFGF; Roche Molecular Biochemicals, Indianapolis, IN) and seeded into 10-cm dishes. All cells were cryopreserved upon reaching confluence. SLA1-1 to SLA1-10 cells were isolated from 10 fetuses at day 28 of pregnancy. Fetuses were mashed through a 60-mesh metal screen (Sigma, St. Louis, MO) using curved surgical forceps slowly so as not to generate excessive heat. The cell suspension was then pelleted and resuspended in 30 ml of DMEM containing 10% FCS, 1 \times nonessential amino acids, 2 ng/ml bFGF, and 10 μ g/ml gentamycin. Cells were seeded in 10-cm dishes, cultured one to three days, and cryopreserved. For transfections, 10 μ g of linearized vector DNA was introduced into 2 million cells by electroporation. Forty-eight hours after transfection, the transfected cells were seeded into 48-well plates at a density of 2,000 cells per well and were selected with 250 μ g/ml of G418 (Gibco-BRL).

Knockout vector construction. Two $\alpha 1,3GT$ knockout vectors, pPL654 and pPL657, were constructed from isogenic DNA of two primary porcine fetal fibroblasts, SLA1-10 and PCFF4-2 cells. A 6.8-kb $\alpha 1,3GT$ genomic fragment, which includes most of intron 8 and exon 9, was generated by PCR from purified

DNA of SLA1-10 cells and PCFF4-2 cells, respectively. The unique *EcoRV* site at the 5' end of exon 9 was converted into a *SaII* site and a 1.8-kb *IRES-neo-poly A* fragment was inserted into the *SaII* site. *IRES* (internal ribosome entry site) functions as a translation initial site for neo protein. Thus, both vectors have a 4.9-kb 5' recombination arm and a 1.9-kb 3' recombination arm (Fig. 1A).

3' PCR and long-range PCR. Approximately 1,000 cells were resuspended in 5 μ l embryo lysis buffer (ELB) (40 mM Tris, pH 8.9, 0.9% Triton X-100, 0.9% Nonidet P-40, 0.4 mg/ml proteinase K), incubated at 65°C for 15 min to lyse the cells, and heated to 95°C for 10 min to inactivate the proteinase K. For 3' PCR analysis, fragments were amplified using the Expand High Fidelity PCR system (Roche Molecular Biochemicals) in 25 μ l reaction volume with the following parameters: 35 cycles of 1 min at 94°C, 1 min at 60°C, and 2 min at 72°C. For LR-PCR, fragments were amplified by using TAKARA LA system (Panvera/Takara, Madison, WI) in 50 μ l reaction volume with the following parameters: 30 cycles of 10 s at 94°C, 30 s at 65°C, 10 min + 20 s increase/cycle at 68°C; and one final cycle of 7 min at 68°C. 3' PCR and LR-PCR conditions for purified DNA was same as for cells except that 1 μ l of purified DNA (30 μ g/ml) was mixed with 4 μ l ELB.

Southern blot analysis of cell samples. Approximately 10⁶ cells were lysed overnight at 60°C in lysis buffer (10 mM Tris, pH 7.5, 10 mM EDTA, 10 mM NaCl, 0.5% (w/v) Sarcosyl, 1 mg/ml proteinase K) and the DNA precipitated with ethanol. The DNA was then digested with *BstEII* and separated on a 1% agarose gel. After electrophoresis, the DNA was transferred to a nylon membrane and probed with the 3'-end digoxigenin-labeled probe. Bands were detected using a chemiluminescent substrate system (Roche Molecular Biochemicals).

Southern blot analysis of pig tissues. Fetal tissues and piglet tails were lysed overnight at 60°C in a shaking incubator with approximately 1 ml lysis solution (50 mM Tris, pH 8.0, 0.15 M NaCl, 10 mM EDTA, 1% SDS, 25% sodium perchlorate, 1% 2-mercaptoethanol, and 200 μ g/ml proteinase K) per 175 mg tissue. DNA was subjected to phenol/chloroform extraction and precipitated with isopropyl alcohol. Resolubilized DNA was treated with RNase A (1 mg/ml) and RNase T1 (1,000 U/ μ l) at 37°C for 1 h, with proteinase K (20 mg/ml) at 55°C for 1 h, then extracted with phenol/chloroform, precipitated with ethanol, and resuspended in TE buffer. About 10 mg DNA was digested with *BstEII* and separated on a 1% agarose gel. Following electrophoresis, the DNA was transferred to a nylon membrane and probed with the 3'-end digoxigenin-labeled probe. Bands were detected using a chemiluminescent substrate system.

Nuclear transfer procedure. Enucleation of *in vitro*-matured oocytes (BioMed, Madison, WI) was begun between 40 and 42 h post-maturation as described previously¹¹. A single fibroblast cell was placed under the zona pellucida in contact with each enucleated oocyte. Fusion and activation were induced by application of an AC pulse of 5 V for 5 s followed by two DC pulses of 1.5 kV/cm for 60 μ s, each using an ECM2001 Electrocell Manipulator (BTX Inc., San Diego, CA). Fused embryos were cultured in NCSU-23 medium for 1–4 h at 38.6°C in a humidified atmosphere of 5% CO₂, and then transferred to the oviduct of an estrus-synchronized recipient gilt. Crossbred gilts (large white/Duroc/landrace) (280–400 lbs) were synchronized as recipients by oral administration of 18–20 mg Regu-Mate (Altrenogest, Hoechst, Warren, NJ) mixed into their feed. Regu-Mate was fed for 14 consecutive days. Human chorionic gonadotropin (hCG, 1,000 units; Intervet America, Millsboro, DE) was administered intramuscularly 105 h after the last Regu-Mate treatment. Embryo transfers were done 22–26 h after the hCG injection.

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Competing interests statement

The authors declare that they have no competing financial interests.

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